

Degradation of Glucose-Dependent Insulinotropic Polypeptide and Truncated Glucagon-Like Peptide 1 *in Vitro* and *in Vivo* by Dipeptidyl Peptidase IV*

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ABSTRACT

The combined actions of glucose-dependent insulinotropic polypeptide (GIP) and truncated glucagon-like peptide-1 (tGLP-1) may fully account for the incretin effect. These hormones are released from the small intestine in response to oral glucose and stimulate insulin release. Recently, evidence has been provided demonstrating the degradation of GIP-(1–42) and GLP-1-(7–36)NH₂ by the serum enzyme dipeptidyl peptidase IV (DPP IV) into the biologically inactive products GIP-(3–42) and GLP-1-(9–36)NH₂. The objective of the current investigation was to develop a method to monitor the degradation of these hormones *in vivo*. Synthetic peptides were radiolabeled and purified by HPLC. Subsequent degradation of the peptides under various conditions was then monitored by further HPLC analysis. Incubation of [¹²⁵I]GIP-(1–42) or [¹²⁵I]GLP-1-(7–36)NH₂ with Wistar rat serum or purified DPP IV resulted in the major N-terminal-truncated products [¹²⁵I]GIP-(3–42) and [¹²⁵I]GLP-1-(9–36)NH₂.

These products were significantly reduced when the specific DPP IV inhibitor diprotin A was included in the incubation mixture and were absent when serum from DPP IV-deficient rats was used. When the labeled peptides were infused into rats at hormone levels within the physiological range, over 50% was metabolized to the truncated forms within 2 min. These products were absent when the tracers were infused into DPP IV-deficient animals. It is concluded that DPP IV may be a primary inactivating enzyme of both GIP and tGLP-1 *in vivo*. As the N-terminal-truncated products of the DPP IV cleavage may not be distinguished from the biologically active hormone by currently employed assays, reports of circulating hormone levels should be reconsidered. The method described in this manuscript may be useful for investigating the durations of action of GIP and tGLP-1 in normal and pathophysiological conditions. (*Endocrinology* 136: 3585–3596, 1995)

IN 1969, UNGER and Eisentraut (1) named the connection between the gut and the pancreatic islets the enteroinsular axis. There are presently two hormones that are widely accepted as candidates for incretins, or hormones of this axis. Glucose-dependent insulinotropic polypeptide (GIP) was purified from extracts of hog duodeno-jejunal mucosa by Brown *et al.* (2, 3), and truncated forms of glucagon-like peptide 1 [GLP-1-(7–36)NH₂ and GLP-1-(7–37); collectively referred to as tGLP-1] were identified from sequencing the preproglucagon gene (4, 5) and later identified in intestinal extracts (6). Both of these hormones are released from the small intestine by nutrients, particularly carbohydrates, and both stimulate insulin secretion in the presence of elevated blood glucose levels (see Refs. 7 and 8 for current reviews). Due to the potential for therapeutic uses of GIP and, more particularly, tGLP-1 in diabetes (9–12), much research has been focused on understanding the mechanisms of their insulinotropic action. Thus, relatively little is presently

known regarding the secretion and metabolism of these hormones.

The circulating half-lives of GIP and tGLP-1 have been estimated generally by determination of hormone immunoreactivity after cessation of an infusion. The half-life of immunoreactive GIP infused into humans averages approximately 20 min (13–16); that of immunoreactive tGLP-1 ranges from 4–11 min (17–19). It appears that the kidney is the major site of GIP clearance (20), with no significant hepatic extraction of endogenous or exogenous GIP (21, 22). Likewise, the kidney plays a major role in the catabolism of tGLP-1 (23, 24).

An important factor that must be considered when examining the metabolism of a hormone is the possibility that hormone measurements associated with immunoreactivity may not necessarily correspond to the biological activity of that hormone. There are numerous proteases in plasma that could potentially render a hormone biologically inactive yet still immunoreactive, as measured by RIA. Thus, although measurements of the half-life of immunoreactive GIP (IRGIP) and immunoreactive tGLP-1 (IRtGLP-1) levels in plasma are useful, they must be combined with studies examining the duration of biological activity of circulating GIP and tGLP-1. The importance of this issue has recently been underscored by the observation that dipeptidyl peptidase IV (DPP IV) hydrolyzes GIP-(1–42) and GLP-1-(7–36)NH₂ in human serum to the respective biologically inactive fragments GIP-(3–42) and GLP-1-(9–36)NH₂ (by cleavage at the penultimate alanine residue) and, thus, may play an impor-

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tant role in the metabolism of these hormones (25). Currently used GIP or tGLP-1 antiserum may be unable to distinguish these truncated N-terminal forms, suggesting that GIP-(3–42) and GLP-1-(9–36)NH₂ probably contribute to IRGIP and IRGLP-1 measurements in the circulation. This manuscript reports on the development of a sensitive method to monitor the degradation of GIP and tGLP-1 by serum *in vitro* and *in vivo*.

Materials and Methods

DPP IV-negative rats

Fischer 344 rats obtained from Japanese Charles River (Kanagawa, Japan) (DPP IV-negative) are DPP IV deficient, whereas Fischer 344 rats from sources in the United States (DPP IV positive) possess normal DPP IV activity (26, 27). The defect in the DPP IV-negative rats appears to be at the level of protein synthesis, because although steady state levels of DPP IV messenger RNA appear normal, abnormal DPP IV is translated, resulting in abnormal posttranslational processing and an absent or nonfunctional protein (28, 29). Two breeding pairs of DPP IV-negative rats were generously supplied by Dr. F. H. Lieblich (Medical College of Georgia, Augusta, GA), and a colony was established at the Department of Physiology, University of British Columbia.

DPP IV assay

To verify the deficiency of DPP IV in the DPP IV-negative rats, a DPP IV assay was established using a slightly modified method from that previously described (30). Blood was collected from the tail of five DPP IV-negative and five DPP IV-positive rats, and the serum was separated. An enzyme substrate was prepared consisting of a solution of 1.4 mM Gly-Pro-*p*-nitroanilide (Sigma Chemical Co., St. Louis, MO) in 114 mM Tris buffer, pH 8.0. Test tubes containing 1 ml Tris buffer were incubated at 37 C, followed by the addition of 100 μ l test serum. The absorbance at 410 nm was then recorded immediately and at 5-min intervals to monitor the appearance of the yellow product of enzyme activity, *p*-nitroaniline. A standard curve was prepared by reading the absorbance of *p*-nitroaniline (Sigma) solutions of 0–1 mM in Tris buffer. The resulting sample absorbance values were then converted to the concentration of *p*-nitroaniline off the standard curve, and the resulting rate of production of *p*-nitroaniline by serum was plotted.

Preparation of [¹²⁵I]GIP AND [¹²⁵I]tGLP-1

To perform *in vivo* studies to investigate the degradation of physiological levels of GIP and tGLP-1, a highly sensitive assay system was required. Radiolabeled GLP-1-(7–36)NH₂ was generously supplied by Novo Nordisk (Copenhagen, Denmark). It was prepared by iodinating GLP-1-(7–36)NH₂ (Novo Nordisk) with Na¹²⁵I using H₂O₂-lactoperoxidase at a neutral pH, and purified by reverse phase HPLC. The specific activity of the resulting purified [¹²⁵I]GLP-1-(7–36)NH₂ was estimated to be ~600 μ Ci/ μ g. Radiolabeled GIP was prepared by iodinating synthetic porcine GIP-(1–42) (Peninsula Laboratories, Belmont, CA) by the chloramine-T method at a neutral pH, as previously described (31). Immediately after iodination, the peak fraction of [¹²⁵I]GIP was purified by reverse phase HPLC on a μ Bondapak C₁₈ column (Waters Associates, Milford, MA). The equipment consisted of two 110B Solvent Delivery Module pumps (Beckman Instruments, San Ramon, CA) controlled by a programmable 421A Controller (Beckman). The radioactivity of the column effluent was measured with a 170 Radioisotope Detector (Beckman) and recorded on a Recordall Series 5000 recorder (Fisher, Vancouver, Canada). Eluant fractions were collected every 0.5 min into 13 $\times$ 10-mm siliconized glass test tubes containing 10 μ l BSA (5%; Sigma RIA grade). Aliquots (10 μ l) of these fractions were counted to ensure recovery and separation, and peak fractions were pooled, frozen at –70 C, lyophilized, and stored at –20 C. The specific activity of the resulting purified [¹²⁵I]GIP was estimated to be 100 μ Ci/ μ g.

HPLC protocols for analysis of [¹²⁵I]GIP and [¹²⁵I]tGLP-1

The HPLC gradient used for the analysis of the degradation of [¹²⁵I]GIP was empirically determined by Verchere (32) to provide separation of the tracer components. The [¹²⁵I]GIP was loaded at a CH₃CN concentration of 32% and eluted by increasing the CH₃CN content to 38% over 10 min and maintaining it at that level for a further 5 min. The column was then washed by increasing the CH₃CN concentration to 60% over 5 min and reequilibrated at 32% for 10 min before injection of another sample. The same HPLC system was used for the analysis of [¹²⁵I]GLP-1-(7–36)NH₂ degradation, with a slightly different gradient. The [¹²⁵I]GLP-1-(7–36)NH₂ was loaded at a CH₃CN concentration of 36% and eluted by increasing the CH₃CN content to 42% over 10 min and maintaining it at that level for a further 5 min. The column was then washed by increasing the CH₃CN concentration to 60% over 5 min and reequilibrated at 36% for 10 min before injection of another sample.

Incubation of [¹²⁵I]GIP and [¹²⁵I]tGLP-1 with serum

To measure the *in vitro* metabolism of [¹²⁵I]GIP-(1–42) and [¹²⁵I]GLP-1-(7–36)NH₂, purified labeled peptide was incubated with serum and subjected to HPLC. Purified label was diluted to 500,000 cpm/50 μ l in 40 mM phosphate buffer (pH 7.0), and 50- μ l aliquots were added to siliconized test tubes containing either 400 μ l (GIP label) or 150 μ l (GLP-1 label) of the same buffer. After gentle mixing, 50 μ l DPP IV-positive or -negative rat serum were added to each tube and incubated at 37 C. Some samples were incubated at 4 C to examine the effects of temperature or with enzyme inhibitors [0.1 mM diprotin A (Sigma), 2% aprotinin (500 kallikrein inhibitor units/ml; Miles Pharmaceuticals, Rexdale, Canada), 50 U/ml bacitracin; Upjohn Co., Kalamazoo, MI], or all three combined as a cocktail. After incubation times ranging from 10 min to 48 h, 100- μ l aliquots were removed and analyzed for breakdown of [¹²⁵I]GIP-(1–42) or [¹²⁵I]GLP-1-(7–36)NH₂, using the HPLC protocols described above.

Incubation of [¹²⁵I]GIP and [¹²⁵I]tGLP-1 with DPP IV

Purified [¹²⁵I]GIP-(1–42) and [¹²⁵I]GLP-1-(7–36)NH₂ were incubated, as described above, with 0.5 mU human placental DPP IV (Calbiochem, La Jolla, CA) and analyzed by HPLC using the above protocols for studying degradation by serum. To further identify the reaction products, a protein sequencer (model 477A, Applied Biosystems, Foster City, CA) was used. Approximately 20,000 cpm of both purified and DPP IV-degraded [¹²⁵I]GIP-(1–42) and [¹²⁵I]GLP-1-(7–36)NH₂ were sequentially digested in the protein sequencer, and the resulting fractions from each cycle were counted. In this fashion, the identity of the proteins was indirectly determined by the position of the [¹²⁵I]tyrosine residues.

In vivo experiments with [¹²⁵I]GIP and [¹²⁵I]tGLP-1

Experiments were next carried out to measure the degradation of [¹²⁵I]GIP-(1–42) or [¹²⁵I]GLP-1-(7–36)NH₂ *in vivo*. Age-matched DPP IV-positive and -negative rats (~350 g) were fasted overnight and anesthetized by ip injection of sodium pentobarbital (60 mg/kg). The right jugular vein was cannulated with PE 90 tubing filled with heparinized saline and attached to a three-way stopcock. The abdomen was then exposed by a midline incision, and the abdominal aorta was cannulated with heparinized saline-filled PE 160 tubing attached to a three-way stopcock. At time zero, ~5 $\times$ 10⁶ cpm purified [¹²⁵I]GIP-(1–42) (~22.5 ng GIP) or [¹²⁵I]GLP-1-(7–36)NH₂ (~3.8 ng tGLP-1) in a volume of 100 μ l 40 mM phosphate buffer (pH 7.0) were injected into the jugular vein, followed by 200–300 μ l heparinized saline. At intervals of 2, 5, 10, and 20 min, 2-ml blood samples were collected from the aorta and transferred immediately to Eppendorf tubes on ice containing diprotin A and aprotinin (final concentration, 0.1 mM and 2%, respectively). Samples were then centrifuged at 7000 $\times$ g for 5 min at 4 C, and the serum was removed and stored on ice.

C₁₈ Sep-Pak cartridges (Waters) were used to purify the [¹²⁵I]GIP or [¹²⁵I]GLP-1 from the collected serum samples. The Sep-Paks were prepared and loaded with sample as instructed by the manufacturer, and after a wash with 20% CH₃CN, samples were eluted with 2 ml 40% CH₃CN into siliconized test tubes containing 10 μ l BSA (5%; Sigma RIA

grade), lyophilized, and stored at -20°C . Each step of the procedure was monitored with a portable γ -detector to ensure maximal recovery of the tracer. Lyophilized samples were then dissolved in $100\ \mu\text{l}$ distilled H_2O and analyzed by HPLC as described above.

Control experiments were performed to ensure that during the sample collection procedure and sample treatment steps, no further degradation of the label occurred. Aliquots of $\sim 3 \times 10^5$ cpm [^{125}I]GIP-(1-42) or [^{125}I]GLP-1-(7-36) NH_2 were incubated with 1 ml serum containing diprotin A (0.1 mM) and aprotinin (2%). Some aliquots were maintained on ice for 1 h, whereas the others were incubated at 37°C for 1 h. Samples were then purified by Sep-Pak, lyophilized, and analyzed by HPLC for any degradation of the [^{125}I]GIP or [^{125}I]GLP-1-(7-36) NH_2 .

Results

DPP IV assay

The standard curve for measurement of DPP IV activity was linear up to a concentration of 0.1 mM. Under the assay conditions used, serum from normal DPP IV-positive rats was able to produce *p*-nitroaniline at a rate of $\sim 1.7\ \mu\text{mol}/\text{min}\cdot\text{ml}$, whereas production with serum from DPP IV-deficient rats occurred at a much slower rate of $\sim 0.1\ \mu\text{mol}/\text{min}\cdot\text{ml}$ (Fig. 1). There was no significant difference in DPP IV activity in the same serum volume from Fischer or Wistar rats (data not shown).

Incubation of purified [^{125}I]GIP with serum

The stability of HPLC-purified [^{125}I]GIP-(1-42) was investigated by incubation at 37°C for up to 24 h in the absence of serum. At intervals of ~ 6 h, label was removed and analyzed by HPLC. After 24-h incubation, only a single component was observed. A single component was also observed after 10-min incubation with 10% Wistar rat serum, but after 1 h, [^{125}I]GIP was resolved into two distinct major components (Fig. 2). One component eluted in the original position (~ 21 – 22 min) and thus consisted of [^{125}I]GIP-(1-42) (peak 1), whereas the new component eluted earlier (~ 19 – 20 min) and

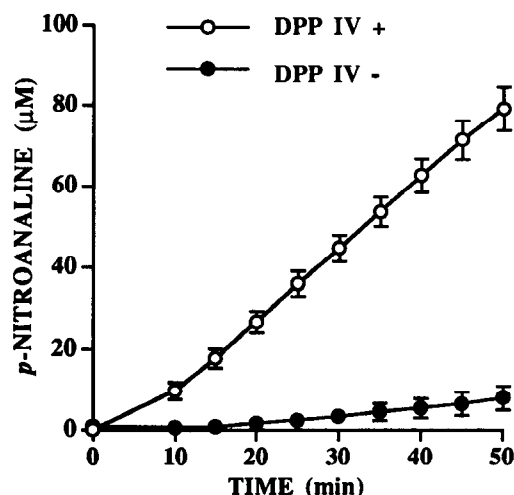


FIG. 1. Determination of relative DPP IV activity in normal (DPP IV +) and DPP IV-deficient (–) rats from rate of production of *p*-nitroaniline. Absorbance values were converted to the concentration of *p*-nitroaniline from a standard curve to obtain the plot of *p*-nitroaniline concentration vs. time. Error bars indicate the SEM ($n = 10$).

was identified to be [^{125}I]GIP-(3-42) (peak 2). With time, as aliquots of incubated [^{125}I]GIP and serum were analyzed by HPLC, a progressive change from [^{125}I]GIP-(1-42) to [^{125}I]GIP-(3-42) was observed (Fig. 2). In contrast, there was no observable production of [^{125}I]GIP-(3-42) when the label was incubated with DPP IV-deficient serum (Fig. 2). However, as with Wistar rat serum, other [^{125}I]GIP fragments with different elution times appeared at a significantly slower rate; the majority eluted at ~ 3 min.

The effect of temperature on the metabolism of [^{125}I]GIP-(1-42) was investigated by performing experiments at 4°C . Both peaks 1 and 2 were observed, indicating that the conversion of [^{125}I]GIP-(1-42) to [^{125}I]GIP-(3-42) occurred (Fig. 3). However, the rate of this reaction (as determined by the changes in the areas of the two peaks with time) was considerably slower. Interestingly, at this temperature, an absence of other peaks was noted, indicating that no further degradation of the tracer had occurred (Fig. 3).

Diprotin A, a competitive inhibitor of DPP IV, was able to significantly reduce the degradation of [^{125}I]GIP-(1-42) to [^{125}I]GIP-(3-42) (raw data not shown). This was evident from the significantly reduced rate of appearance of peak 2 and the reduction in peak 1 when 0.1 mM diprotin A was added to the serum containing [^{125}I]GIP. However, this inhibitor did not alter the degradation of [^{125}I]GIP to other fragments, with considerable radioactivity eluting at 3 min as incubation progressed. In contrast, the protease inhibitor aprotinin had no significant effect on the conversion of [^{125}I]GIP-(1-42) to [^{125}I]GIP-(3-42), as evident from the reduction of peak 1 and production of peak 2 with time by Wistar rat serum in the presence of 2% aprotinin (raw data not shown). However, this inhibitor did reduce the rate of production of other peaks. The antibiotic bacitracin (50 U/ml) significantly reduced the conversion of [^{125}I]GIP-(1-42) to [^{125}I]GIP-(3-42) by serum, but not to the same degree as diprotin A (raw data not shown). Bacitracin, however, greatly reduced the further fragmentation of [^{125}I]GIP, as indicated by the near absence of other peaks. When used in combination, these inhibitors effectively reduced the degradation of [^{125}I]GIP (data not shown). After 20-h incubation of [^{125}I]GIP with Wistar rat serum and the inhibitor cocktail at 37°C , $\sim 35\%$ existed as [^{125}I]GIP-(3-42) (peak 2), and the remainder as [^{125}I]GIP-(1-42) (peak 1).

Incubation of purified [^{125}I]tGLP-1 with serum

The stability of HPLC-purified [^{125}I]GLP-1-(7-36) NH_2 was investigated by incubation at 37°C for up to 48 h in the absence of serum. At 6-h intervals, label was removed and analyzed by HPLC. Even after 48 h, a single component was observed (Fig. 4). After incubation with 20% Wistar rat serum for 30 min, [^{125}I]tGLP-1 could be resolved into two components. One component eluted in the original position (~ 17 – 18 min) and thus consisted of [^{125}I]GLP-1-(7-36) NH_2 (peak 1), whereas the new component (peak 2) eluted later (~ 20 min) and was identified as [^{125}I]GLP-1-(9-36) NH_2 . With time, as aliquots of incubated [^{125}I]GLP-1-(7-36) NH_2 and serum were analyzed by HPLC, a progression from [^{125}I]GLP-1-(7-36) NH_2 to [^{125}I]GLP-1-(9-36) NH_2 was observed (Fig. 4). In contrast, there was little degradation of the GLP-1-(7-36) NH_2 tracer to [^{125}I]GLP-1-(9-36) NH_2 with serum from DPP IV-deficient animals (Fig. 4).

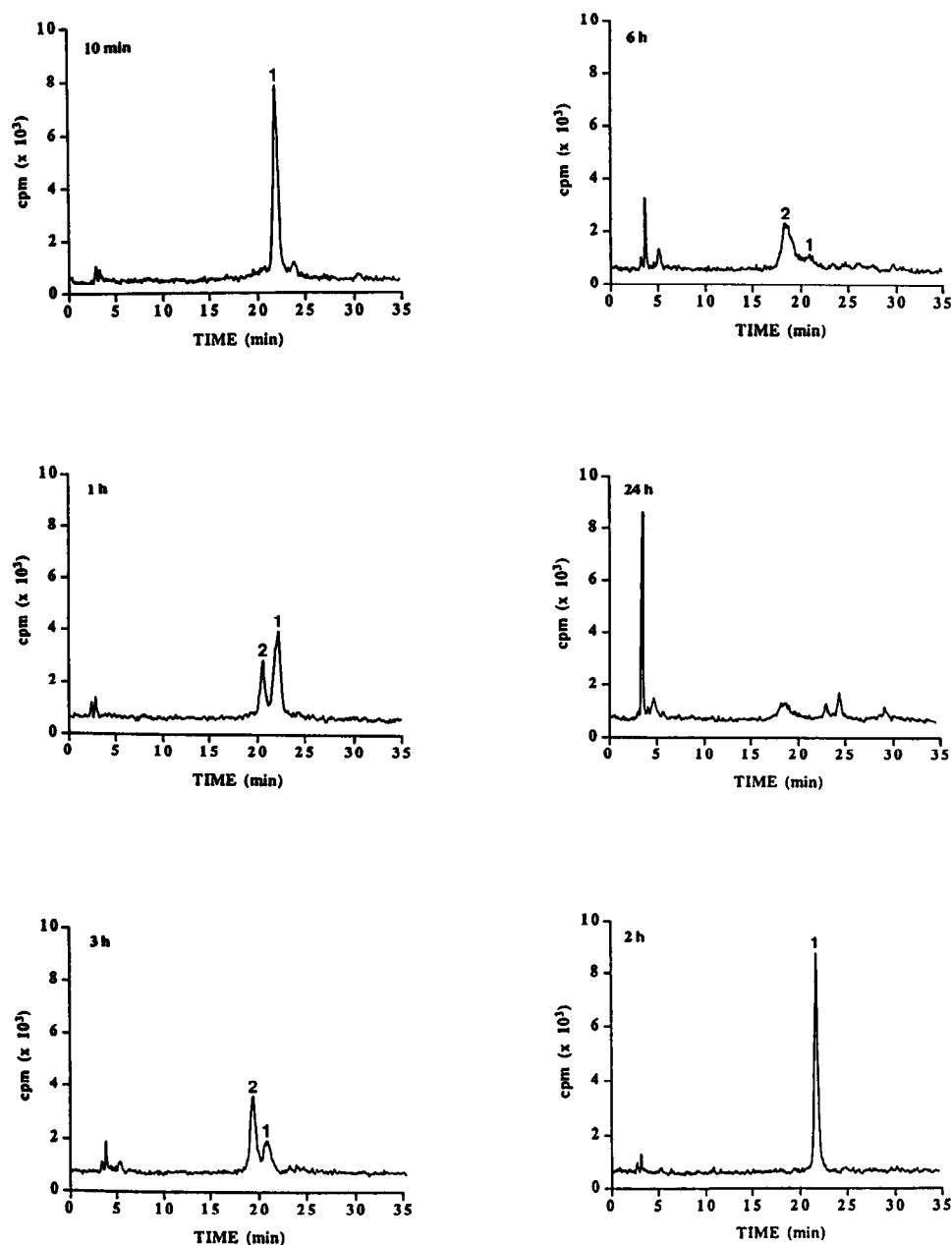


FIG. 2. HPLC elution profiles of [^{125} I]GIP ($\sim 10^5$ cpm) incubated with Wistar rat serum (10%) at 37 C for periods ranging from 10 min to 24 h or with DPP IV (–) rat serum (10%) for 2 h (bottom right plot). Peak 1 is [^{125} I]GIP-(1–42), peak 2 corresponds to [^{125} I]GIP-(3–42), and other peaks consist of unidentified [^{125} I]GIP fragments.

There were, however, appreciable quantities of other degradation products after 8.5-h incubation, which eluted in identical positions to products formed in Wistar serum at the later time points.

The effect of temperature on the degradation of [^{125} I]tGLP-1 was investigated by performing the same experiments at 4 C. As observed with [^{125} I]GIP-(1–42), [^{125} I]GLP-1-(7–36)NH₂ was still degraded at 4 C, albeit at a much slower rate. Both peaks 1 and 2 could be observed, indicating that the conversion of [^{125} I]GLP-1-(7–36)NH₂ to [^{125} I]GLP-1-(9–36)NH₂ was still occurring (Fig. 5). It is interesting to note that, as with the GIP tracer, no other degradation products of [^{125} I]GLP-1-(7–36)NH₂ incubated with Wistar serum at 4 C were observed, even after 69 h.

The effect of diprotin A on the degradation of [^{125} I]GLP-

1-(7–36)NH₂ was also investigated. In the presence of this DPP IV inhibitor, degradation of [^{125} I]GLP-1-(7–36)NH₂ (peak 1) to [^{125} I]GLP-1-(9–36)NH₂ (peak 2) was greatly reduced, with the majority still eluting as [^{125} I]GLP-1-(7–36)NH₂ after 7-h incubation with Wistar rat serum (raw data not shown). However, diprotin A had no effect on reducing the formation of other degradation products, as evident from the presence of other significant components by 7 h of incubation. The enzyme inhibitor bacitracin was effective at reducing the degradation of GLP-1-(7–36)NH₂ label by serum, with significant quantities of [^{125} I]GLP-1-(7–36)NH₂ remaining after 11 h (raw data not shown). In the presence of bacitracin, the major enzymatic product appeared to be [^{125} I]GLP-1-(9–36)NH₂ (peak 2), with relatively little further degradation occurring. Aprotinin, on the other hand, clearly

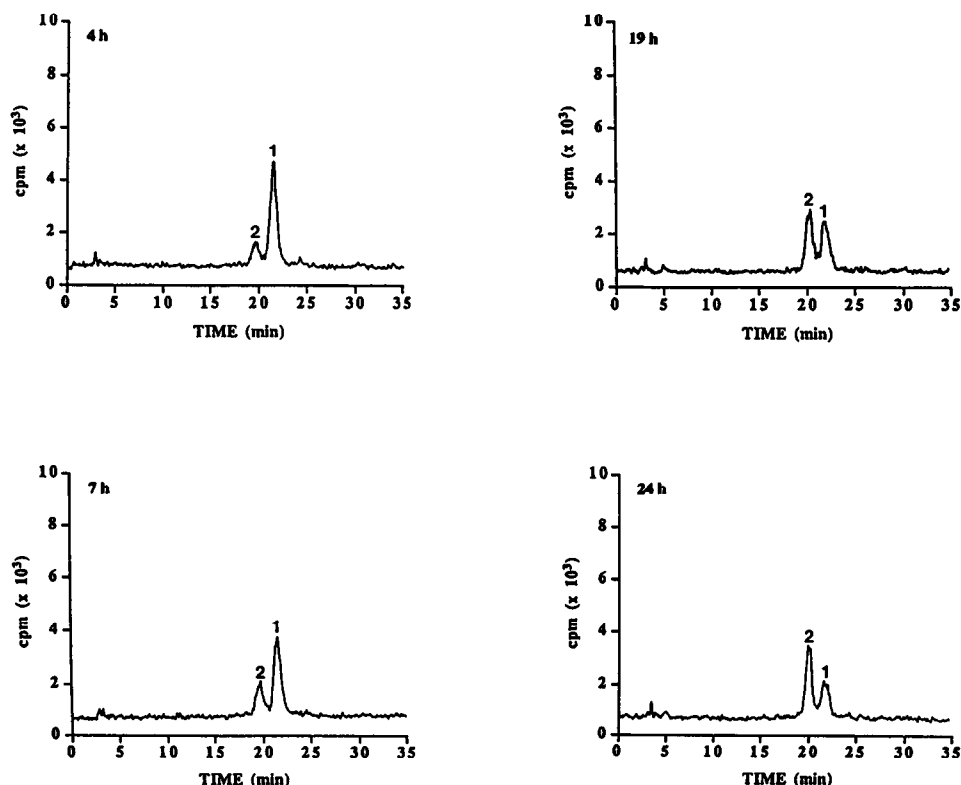


FIG. 3. HPLC elution profiles of [^{125} I]GIP ($\sim 10^5$ cpm) incubated with Wistar rat serum (10%) at 4 C. Peak 1 is [^{125} I]GIP-(1-42), and peak 2 corresponds to [^{125} I]GIP-(3-42).

had no effect on the degradation of the GLP-1-(7-36)NH₂ tracer to [^{125} I]GLP-1-(9-36)NH₂ (raw data not shown). The conversion of [^{125} I]GLP-1-(7-36)NH₂ to [^{125} I]GLP-1-(9-36)NH₂ was virtually complete by 5 h. However, in the presence of this enzyme inhibitor, there was no further degradation, as indicated by the absence of significant quantities of other components that could be separated by HPLC. When used in combination, bacitracin, diprotin A, and aprotinin were highly effective in reducing the degradation of the labeled GLP-1-(7-36)NH₂ (data not shown).

Incubation of [^{125} I]GIP and [^{125} I]tGLP-1 with DPP IV

To verify a direct role for DPP IV in the degradation of both [^{125} I]GIP-(1-42) and [^{125} I]GLP-1-(7-36)NH₂, these labeled peptides were incubated with a purified preparation of DPP IV. With both peptides there was a time-dependent breakdown to the primary products [^{125} I]GIP-(3-42) and [^{125} I]GLP-1-(9-36)NH₂, respectively (Fig. 6). No significant quantities of other products were resolved. To provide further verification for the identity of the enzymatic products, a protein sequencer was used. The amount of protein loaded was insufficient for direct determination of the amino acid sequences, but the sequentially removed residues were collected and counted. For [^{125} I]GIP-(1-42), a minor amount of radioactivity was found on residue 1, and a major peak at residue 10, consistent with known tyrosines at these positions that are targets for iodination. After incubation with human placental DPP IV and resequencing, a new peak of radioactivity was generated at cycle 8, consistent with the formation of N-terminally truncated [^{125} I]GIP-(3-42) (Fig. 7A). For [^{125} I]GLP-1-(7-36)NH₂, a peak of radioactivity was found on residue 13, consistent with the location of tyrosine

at this position. After incubation with human placental DPP IV and resequencing, a new peak of radioactivity was observed in cycle 11, consistent with the formation of N-terminally truncated [^{125} I]GLP-1-(9-36)NH₂ (Fig. 7B).

Summary of [^{125} I]GIP and [^{125} I]tGLP-1 degradation *in vitro*

A summary of the rate of breakdown of [^{125} I]GIP is presented in Fig. 8A. The relative proportion of intact [^{125} I]GIP-(1-42) to degradation products was calculated by expressing the area of the [^{125} I]GIP-(1-42) (peak 1) as a percentage of the total peak area. The time required for degradation of 50% of the [^{125} I]GIP-(1-42) was ~ 2.2 h in 10% normal Wistar rat serum. This time was significantly increased to 5.6 h by the addition of bacitracin and to 16.1 h by storage at 4 C. The addition of diprotin A lengthened the time required to degrade 50% of the [^{125} I]GIP-(1-42) to an estimated 29 h. No degradation of [^{125} I]GIP-(1-42) to [^{125} I]GIP-(3-42) was observed in DPP IV-deficient rat serum.

Plots of the rate of degradation of [^{125} I]GLP-1-(7-36)NH₂ by serum were also made (Fig. 8B). The time required for degradation of 50% of the [^{125} I]GLP-1-(7-36)NH₂ was ~ 1.7 h in 20% normal Wistar rat serum. This time was significantly increased to ~ 5.2 h by the addition of bacitracin and to an estimated 15 h by the addition of diprotin A. The time required for 50% of the tGLP-1 label to be degraded by serum incubated at 4 C was too long to be accurately estimated. No significant degradation of [^{125} I]GLP-1-(7-36)NH₂ to [^{125} I]GLP-1-(9-36)NH₂ was observed in DPP IV-deficient rat serum.

A summary of the rate of degradation of [^{125} I]GIP-(1-42) and [^{125} I]GLP-1-(7-36)NH₂ by purified DPP IV is illustrated

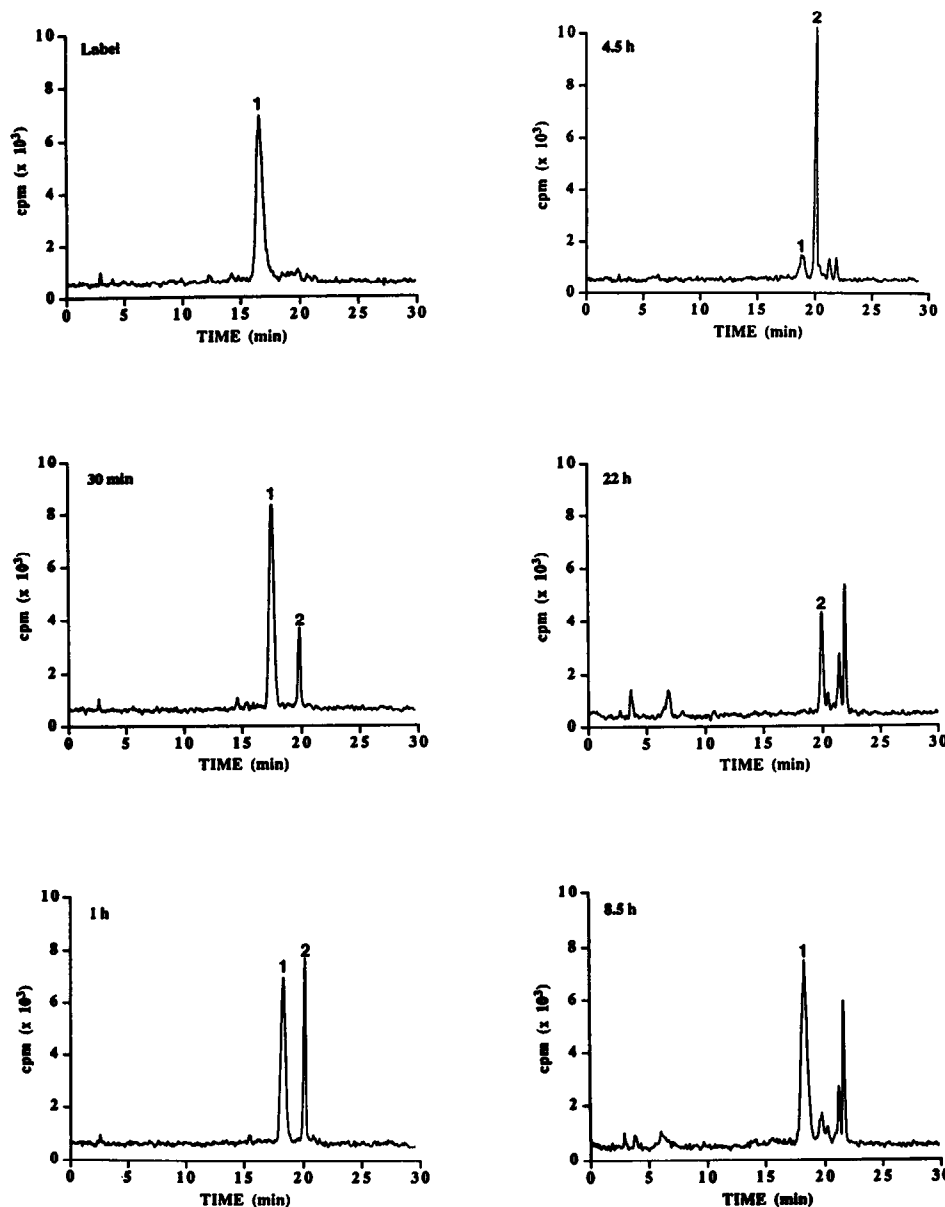


FIG. 4. HPLC elution profiles of $[^{125}\text{I}]\text{GLP-1-(7-36)NH}_2$ ($\sim 10^5$ cpm) incubated alone at 37 C for 48 h (label), with Wistar rat serum (20%) for periods ranging from 30 min to 22 h, or with DPP IV (-) rat serum (20%) for 8.5 h (bottom right plot). Peak 1 is $[^{125}\text{I}]\text{GLP-1-(7-36)NH}_2$, peak 2 corresponds to $[^{125}\text{I}]\text{GLP-1-(9-36)NH}_2$, and other peaks consist of unidentified $[^{125}\text{I}]\text{GLP-1}$ fragments.

in Fig. 8C. The times required for degradation of 50% of the $[^{125}\text{I}]\text{GIP-(1-42)}$ or $[^{125}\text{I}]\text{GLP-1-(7-36)NH}_2$ were 20.5 and 7.5 h, respectively.

Analysis of $[^{125}\text{I}]\text{GIP}$ and $[^{125}\text{I}]\text{tGLP-1}$ degradation *in vivo*

The degradation of GIP and tGLP-1 *in vivo* was investigated by HPLC analysis of Sep-Pak-extracted aliquots of plasma at different times after injection. Based on the *in vitro* results, it was expected that $[^{125}\text{I}]\text{GIP}$ and $[^{125}\text{I}]\text{tGLP-1}$ would be rapidly degraded in the circulation, and rapid delivery of the tracer and expeditious blood sampling were deemed necessary. Labeled peptides were, therefore, injected through a jugular venous cannula, and plasma samples were collected directly from the aorta. It was also imperative that any further degradation subsequent to sample collection be prevented. Based on the *in vitro* results, it was determined that samples should be collected into tubes on ice containing

diprotin A and aprotinin (final concentrations, 0.1 mM and 2%, respectively) to keep degradation to a minimum. Subsequent to collection, the plasma samples were then purified by Sep-Pak as described above. The recovery of tracer from plasma collection to lyophilization was approximately 80%. HPLC analysis of control samples of $[^{125}\text{I}]\text{GIP}$ or $[^{125}\text{I}]\text{tGLP-1}$ incubated with serum and the inhibitors revealed no detectable degradation, indicating that experimental samples were treated appropriately.

The degradation of both labeled peptides occurred very quickly *in vivo*. By 2 min, over 50% of the $[^{125}\text{I}]\text{GIP-(1-42)}$ (peak 1) was degraded to $[^{125}\text{I}]\text{GIP-(3-42)}$ (peak 2), and by 10 min, $\sim 91\%$ of the $[^{125}\text{I}]\text{GIP}$ was $[^{125}\text{I}]\text{GIP-(3-42)}$, with no other degradation products discernible (Fig. 9). In contrast, there was no degradation of $[^{125}\text{I}]\text{GIP}$ infused into DPP IV-deficient rats by 10 min (Fig. 9). The rate of degradation of $[^{125}\text{I}]\text{GLP-1-(7-36)NH}_2$ was, likewise, extremely fast *in vivo*.

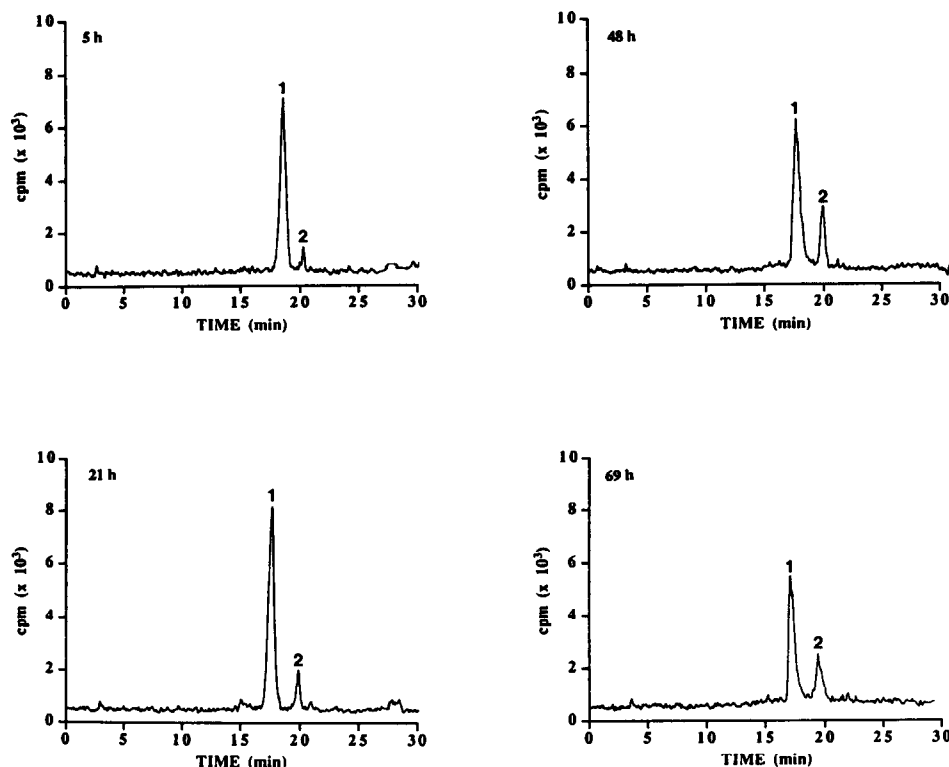


FIG. 5. HPLC elution profiles of $[^{125}\text{I}]\text{GLP-1-(7-36)NH}_2$ ($\sim 10^5$ cpm) incubated with Wistar rat serum (20%) at 4 C. Peak 1 is $[^{125}\text{I}]\text{GLP-1-(7-36)NH}_2$, and peak 2 corresponds to $[^{125}\text{I}]\text{GLP-1-(9-36)NH}_2$.

Once again, by 2 min, greater than 50% of the $[^{125}\text{I}]\text{GLP-1-(7-36)NH}_2$ was converted to $[^{125}\text{I}]\text{GLP-1-(9-36)NH}_2$ (peak 2; Fig. 10). By 10 min, there was no discernible peak 1, but there was a major peak 2, the $[^{125}\text{I}]\text{GLP-1-(9-36)NH}_2$. At this time point, there were small, but significant, quantities of subsequent degradation products observable. As with the $[^{125}\text{I}]\text{GIP}$ *in vivo* experiments, there was no observable degradation of $[^{125}\text{I}]\text{GLP-1-(7-36)NH}_2$ to $[^{125}\text{I}]\text{GLP-1-(9-36)NH}_2$ in the DPP IV-deficient animals (Fig. 10).

Discussion

In 1981, Jörnvall and colleagues (33) identified the GIP fragment (3–42, lacking Tyr-Ala) as a contaminant of GIP preparations purified from porcine duodenal mucosal extracts. These researchers suggested that the GIP fragment, which constituted more than 20% of that particular batch of purified GIP, might be formed by degradation by intestinal enzymes, including dipeptidyl aminopeptidase. Concomitantly, Brown *et al.* (34) demonstrated that this large peptide fragment was neither insulintropic nor somatostatinotropic, two properties characteristic of the parent molecule. As GIP belongs to a superfamily of peptides sharing sequence homology, it could be predicted that such an enzyme would act on other hormones with similar N-terminal amino acid sequences. Buckley and Lundquist (35) noted that a second member of the glucagon family, tGLP-1, was degraded by an amino dipeptidase constituent of plasma by removal of the N-terminal dipeptide (His-Ala). Frohman *et al.* (36) previously observed that GRF [GRF-(1–44)] was cleaved to 3–44 by an enzyme in plasma. The N-terminal amino acids (1 and 2) of GRF are identical to those in GIP

(Tyr-Ala). Furthermore, GRF-(3–44), like GIP-(3–42), was biologically inactive. The plasma enzyme responsible was later identified as DPP IV (37). They clearly demonstrated that this enzyme was present in human plasma and quickly inactivated GRF *in vivo*. DPP IV has since been confirmed as being at least partially responsible for the degradation of the incretins GIP and tGLP-1 in plasma (25).

The enzyme DPP IV preferentially cleaves peptides and proteins with X-Pro, X-Hyp, or X-Ala at their N-terminals (38) and, to a lesser extent, X-Ser, X-Thr, and X-Val, where X refers to any amino acid (39). It was first identified in rat liver (40) and has since been found ubiquitously distributed throughout mammalian tissues, with highest activity in kidney and intestinal brush-border membrane, as well as in insects, bacteria, and yeast (see Ref. 41 for review). In keeping with the widespread occurrence of DPP IV, evidence suggests that the enzyme is involved in a number of biochemical processes. This list includes renal transport and intestinal digestion of proline-containing peptides, immunological activation of immunocompetent cells, and fibronectin-mediated adhesion (41). DPP IV also appears to have a role in the inactivation of biologically relevant peptides (25, 37, 42), and it is the involvement of DPP IV in the metabolism of GIP and tGLP-1 that was the focus of research discussed in this manuscript.

Confirmation of a physiological role for DPP IV in the metabolism of GIP and tGLP-1 requires *in vivo* evidence. Frohman *et al.* (36) proposed a role for DPP IV in GRF metabolism by demonstrating degradation of GRF-(1–44) to GRF-(3–44) *in vivo*. These observations were accomplished by injecting subjects with GRF and analyzing serum by HPLC. There were, however, several disadvantages to this

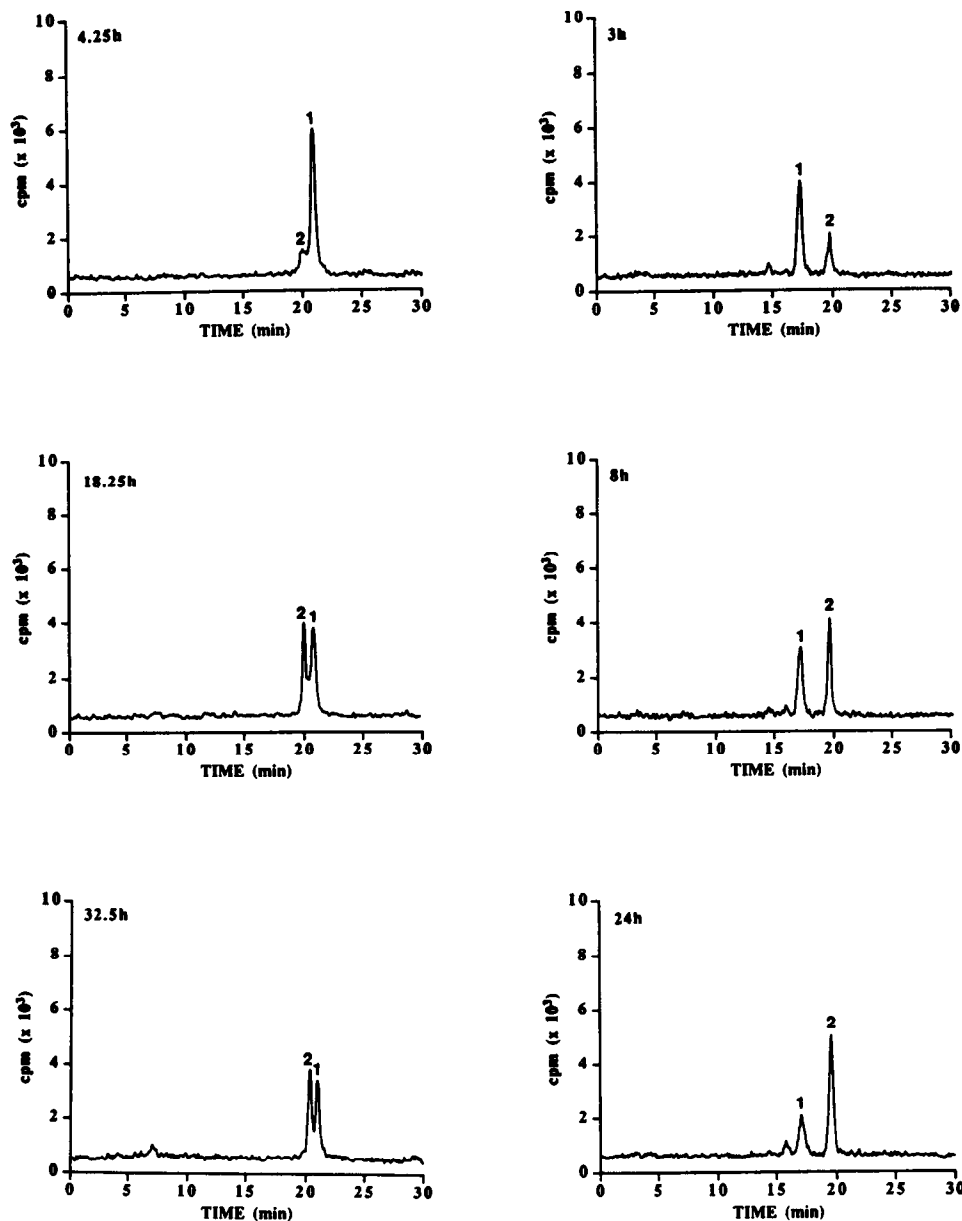


FIG. 6. HPLC elution profiles of [¹²⁵I]GIP (~10⁵ cpm; *left plots*) and [¹²⁵I]GLP-1-(7-36)NH₂ (~10⁵ cpm; *right plots*) incubated with 0.5 mU human placental DPP IV. In the *left plots*, peak 1 is [¹²⁵I]GIP-(1-42), and peak 2 corresponds to [¹²⁵I]GIP-(3-42). In the *right plots*, peak 1 is [¹²⁵I]GLP-1-(7-36)NH₂, and peak 2 corresponds to [¹²⁵I]GLP-1-(9-36)NH₂.

system, one being the requirement of infusing supraphysiological doses of hormone. Subjects were injected with GRF at 1 µg/kg, and even at this dose, plasma concentrations were well below the concentrations required for UV detection after HPLC, thus necessitating the collection of eluted fractions and analysis by RIA. Furthermore, the interpretation of these data was made difficult by the presence of endogenous hormone. A primary objective of the present investigation was, therefore, to develop a sensitive method to detect the conversion of GIP-(1-42) to GIP-(3-42) and that of GLP-1-(7-36)NH₂ to GLP-1-(9-36)NH₂ *in vivo* to support the *in vitro* results.

It was hypothesized that the required assay sensitivity could be achieved using radiolabeled peptides as DPP IV substrates, followed by HPLC analysis coupled with γ-radiation detection. *In vitro* experiments were first performed to confirm that the purified [¹²⁵I]GIP and [¹²⁵I]t-

GLP-1 would still serve as substrates for DPP IV, and that the products of enzyme degradation could be readily resolved and quantified. These experiments with Wistar rat serum clearly demonstrated the time-dependent metabolism of [¹²⁵I]GIP-(1-42) and [¹²⁵I]GLP-1-(7-36)NH₂. Furthermore, based on the specific activity of the tracers, peaks equivalent to as little as 5 pg GIP-(1-42) or 0.8 pg GLP-1-(7-36)NH₂ could be identified, verifying the sensitivity of the method.

As observed by Mentlein *et al.* (25), the metabolism of GIP and tGLP-1 yielded one primary product in each case. Consistent with the findings of Mentlein *et al.* (25), results from protein sequencer cycles indicated that [¹²⁵I]GIP-(1-42) was degraded to [¹²⁵I]GIP-(3-42), whereas [¹²⁵I]GLP-1-(7-36)NH₂ was reduced to [¹²⁵I]GLP-1-(9-36)NH₂. The times required for conversion of 50% of [¹²⁵I]GIP-(1-42) to [¹²⁵I]GIP-(3-42) or of [¹²⁵I]GLP-1-(7-36)NH₂ to [¹²⁵I]GLP-1-

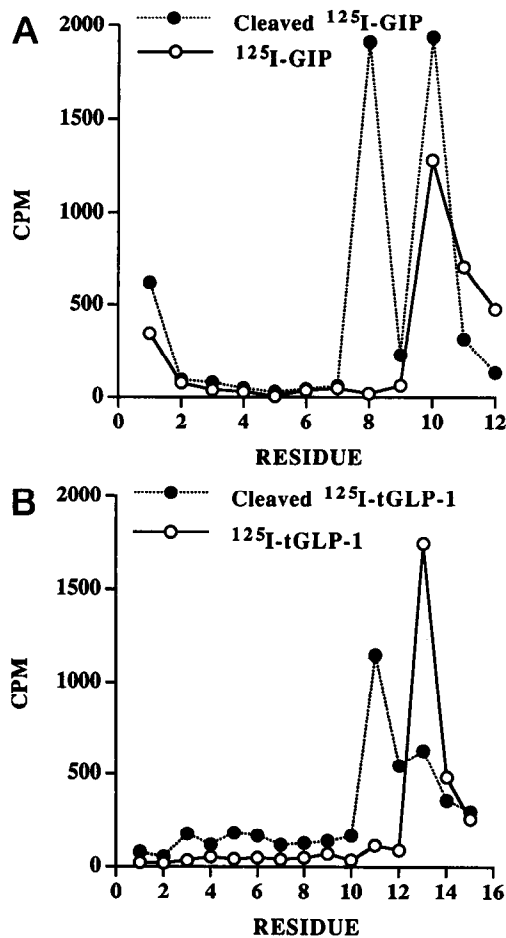


FIG. 7. Graphs showing the radioactivity (counts per min) of N-terminal residues removed by sequential cycles in a protein sequencer of A) $[^{125}\text{I}]\text{GIP}$ -(1-42) and $[^{125}\text{I}]\text{GIP}$ -(1-42) after 24-h incubation with human placental DPP IV (cleaved), and B) $[^{125}\text{I}]\text{GLP-1}$ -(7-36) NH_2 and $[^{125}\text{I}]\text{GLP-1}$ -(7-36) NH_2 after 24-h incubation with human placental DPP IV (cleaved).

(9-36) NH_2 *in vitro* in rat serum under slightly different assay conditions were estimated to be 2.2 and 1.7 h, respectively. Other peaks appeared with further incubation, indicating further degradation of the tracers, but these reactions occurred at much slower rates. Further evidence that the primary products were $[^{125}\text{I}]\text{GIP}$ -(3-42) and $[^{125}\text{I}]\text{GLP-1}$ -(9-36) NH_2 was provided by the observations that the inclusion of the DPP IV inhibitor, diprotin A, significantly reduced the rates of production of these peaks. Furthermore, rats specifically lacking DPP IV, served as suitable controls, and incubation of labeled peptides with serum from these animals yielded no significant amount of $[^{125}\text{I}]\text{GIP}$ -(3-42) or $[^{125}\text{I}]\text{GLP-1}$ -(9-36) NH_2 . The same primary products were observed when $[^{125}\text{I}]\text{GIP}$ or $[^{125}\text{I}]\text{GLP-1}$ -(7-36) NH_2 was incubated with a purified preparation of DPP IV. The significantly slower reaction rate achieved with the enzyme compared to diluted serum may indicate that DPP IV requires cofactors for optimal activity, which may normally be present in serum. However, the addition of serum from DPP IV-deficient rats did not improve the efficiency of the purified DPP IV (data not shown).

This sensitive model also allowed for further character-

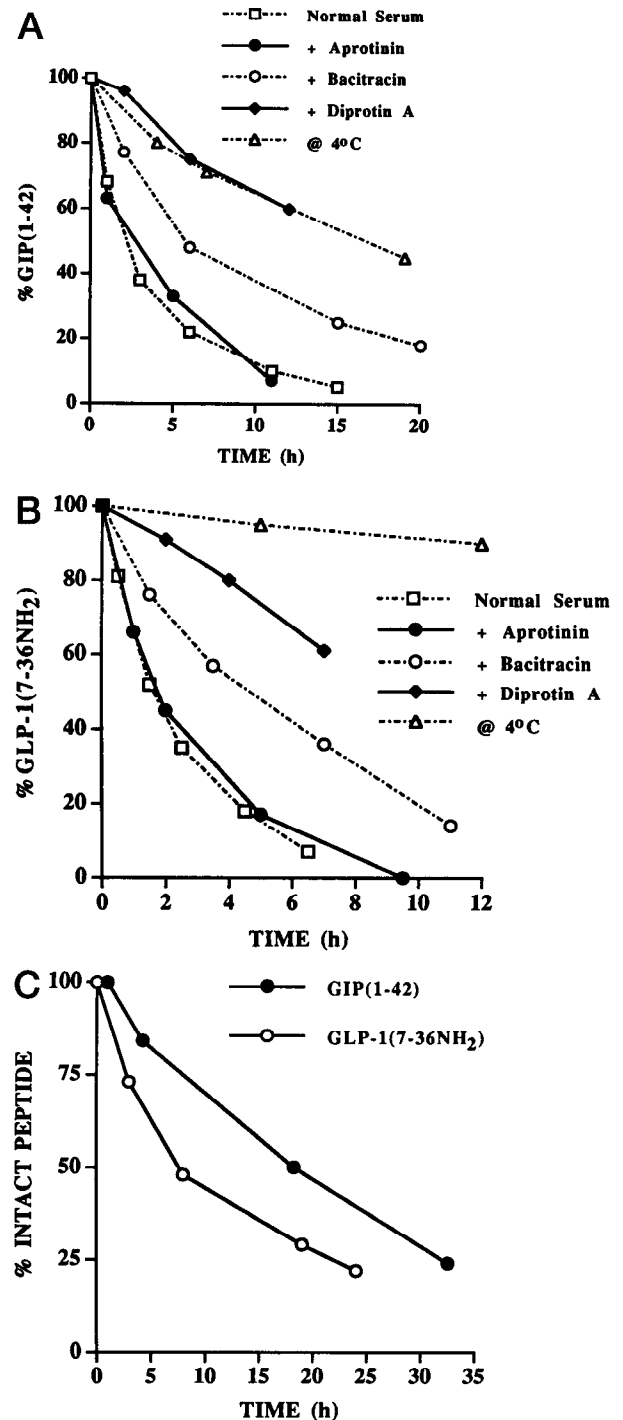
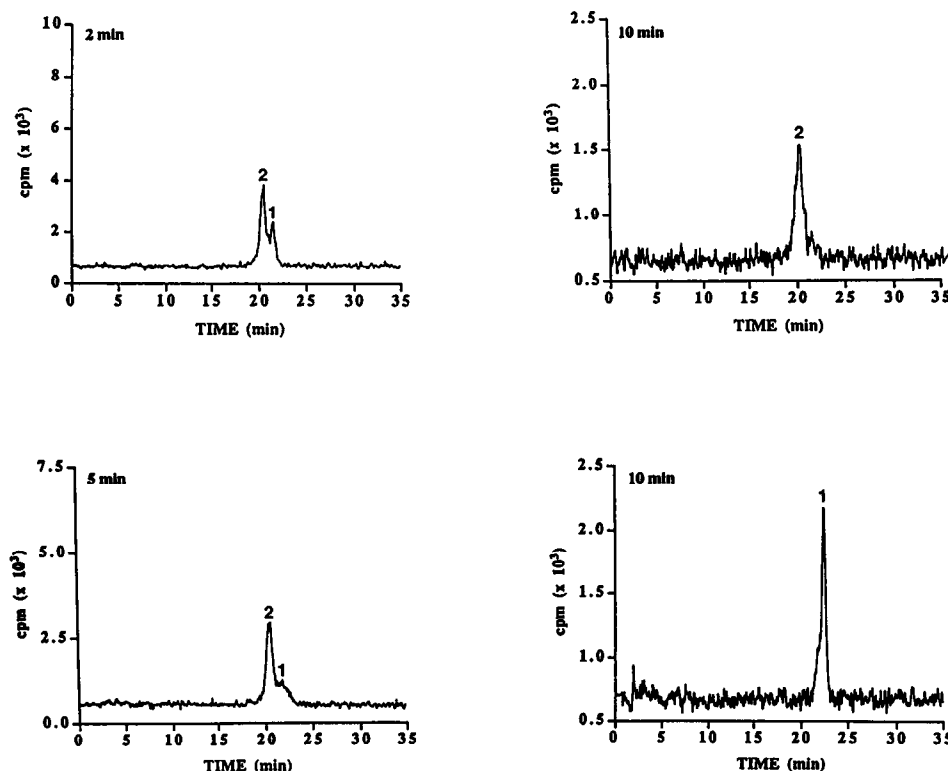


FIG. 8. Graphs summarizing the *in vitro* degradation rate of (A) $[^{125}\text{I}]\text{GIP}$ -(1-42) in rat serum (10%), and (B) $[^{125}\text{I}]\text{GLP-1}$ -(7-36) NH_2 in rat serum (20%), with the addition of aprotinin (2%), bacitracin (50 U/ml), or diprotin A (0.1 mM) or with reduced temperature (37 to 4°C). C, Graph summarizing the degradation rates of $[^{125}\text{I}]\text{GIP}$ -(1-42) and $[^{125}\text{I}]\text{GLP-1}$ -(7-36) NH_2 (intact peptide) *in vitro* incubated at 37°C with 0.5 mU purified DPP IV.

ization of the metabolism of $[^{125}\text{I}]\text{GIP}$ -(1-42) and $[^{125}\text{I}]\text{GLP-1}$ -(7-36) NH_2 . The inhibitor aprotinin had no effect on the production of $[^{125}\text{I}]\text{GIP}$ -(3-42) or $[^{125}\text{I}]\text{GLP-1}$ -(9-36) NH_2 . Aprotinin, however, significantly reduced subsequent me-

FIG. 9. HPLC elution profiles of [^{125} I]GIP-(1-42) infused into a Wistar rat (left plots and top right plot) and into a DPP IV-deficient rat (bottom right 10 min plot) collected in 2 ml serum with diprotin A (0.1 mM) and aprotinin (2%), purified by Sep-Pak, lyophilized, and reconstituted, as described in *Materials and Methods*. In the left plots, $\sim 8 \times 10^4$ cpm were loaded; in the right plots, $\sim 4 \times 10^4$ cpm were loaded. Peak 1 is [^{125} I]GIP-(1-42), and peak 2 corresponds to [^{125} I]GIP-(3-42).



tabolism to other unidentified products, probably by inhibiting the action of other enzymes, such as trypsin. Buckley and Lundquist (35) reported that bacitracin was an effective inhibitor of enzymatic cleavage of GLP-1-(7-36)NH₂, and therefore, this inhibitor was also investigated. Bacitracin significantly reduced the degradation of [^{125} I]GIP-(1-42) to [^{125} I]GIP-(3-42) and that of [^{125} I]GLP-1-(7-36)NH₂ to [^{125} I]GLP-1-(9-36)NH₂, but not to the degree observed with diprotin A. However, although diprotin A did not appear to significantly affect subsequent label degradation, bacitracin was highly effective in this regard. In combination, diprotin A, aprotinin, and bacitracin provide highly effective protection against [^{125} I]GIP or [^{125} I]GLP-1-(7-36)NH₂ degradation.

Interestingly, incubation with serum at 4 C resulted in significant degradation of [^{125} I]GIP-(1-42) to [^{125} I]GIP-(3-42) and of [^{125} I]GLP-1-(7-36)NH₂ to [^{125} I]GLP-1-(9-36)NH₂, although the rates were much slower than those of the same reactions at 37 C. Other enzymes capable of metabolizing GIP or GLP-1-(7-36)NH₂ were completely ineffective at this temperature, however, as indicated by the lack of other significant peaks (products) even after 24-h (GIP) or 69-h [GLP-1-(7-36)NH₂] incubation. Therefore, in addition to including the enzyme inhibitor cocktail, these results suggest that storage at 4 C is an effective method to reduce degradation of GIP and tGLP-1.

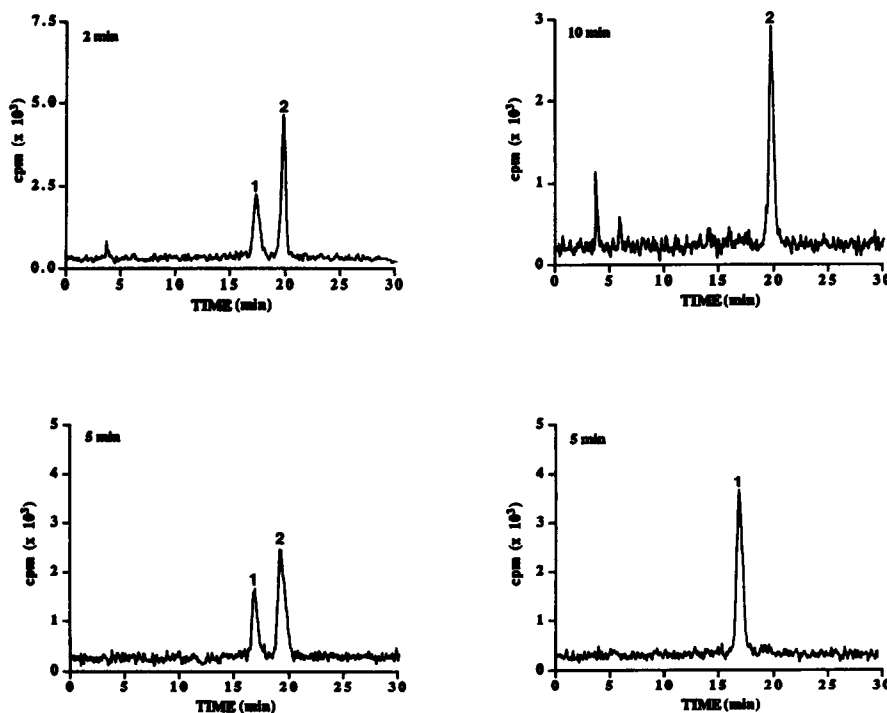
This protocol thus appeared to fulfill the requirements of a suitable assay for the metabolism of GIP or tGLP-1, and therefore, *in vivo* experiments were performed. Approximately 5×10^6 cpm purified [^{125} I]GIP-(1-42) (~ 22.5 ng GIP) or [^{125} I]GLP-1-(7-36)NH₂ (~ 3.8 ng tGLP-1) were injected into each rat. Thus, for a plasma volume of ~ 10 ml, the peptide concentrations were estimated to be ~ 2.25 and ~ 0.38 ng/ml, respectively, values within the physiological range. This

method thus allows the use of concentrations at least 10-fold lower than those used by Frohman *et al.* (36) in the investigation of *in vivo* metabolism of GRF. As expected, the rates of [^{125} I]GIP and [^{125} I]GLP-1-(7-36)NH₂ degradation were much more rapid *in vivo* than those observed *in vitro* with diluted serum. Only ~ 2 min were required for more than half of the [^{125} I]GIP-(1-42) to be converted to [^{125} I]GIP-(3-42) or for [^{125} I]GLP-1-(7-36)NH₂ to be converted to [^{125} I]GLP-1-(9-36)NH₂. These results are comparable to those reported by Frohman *et al.* (36), who found that 1 min after injection of 1 μ g/kg GRF-(1-44), $43 \pm 7\%$ of the total immunoreactive GRH was GRH-(3-44). This short half-life of DPP IV substrates is probably a reflection of the fact that DPP IV is found in high concentrations as an ectoenzyme of the plasma membrane of numerous cell types, in addition to plasma (41).

There are many implications of these results that should be considered. The data presented suggest that DPP IV is a primary enzyme involved in the degradation of GIP and tGLP-1 *in vivo*. The products of these reactions [GIP-(3-42) and GLP-1-(9-36)NH₂] appear to be biologically inactive (34, 43-47). This rapid hormone inactivation in conjunction with the well documented glucose dependency of GIP and tGLP-1 action may act to ensure the prevention of hyperinsulinemia and subsequent hypoglycemia. There have been no assays reported to date using antisera that can distinguish between GIP-(1-42) and GIP-(3-42) or between GLP-1-(7-36)NH₂ and GLP-1-(9-36)NH₂. Therefore, as GIP-(3-42) and GLP-1-(9-36)NH₂ are biologically inactive, yet still immunoreactive, reports of circulating IRGIP and IRtGLP-1 levels in health and disease must be reconsidered.

Overactivity of DPP IV could result in increased metabolism of the insulinotropic hormones GIP and tGLP-1 and, thus, a

FIG. 10. HPLC elution profiles of [^{125} I]GLP-1-(7-36)NH₂ infused into a Wistar rat (left plots and top right plot) and into a DPP IV-deficient rat (bottom right 5 min plot), collected in 2 ml serum with diprotin A (0.1 mM) and aprotinin (2%), purified by Sep-Pak, lyophilized, and reconstituted, as described in *Materials and Methods*. In the left plots and the bottom right plot, $\sim 8 \times 10^4$ cpm were loaded; in the top right plot, $\sim 4 \times 10^4$ cpm were loaded. Peak 1 is [^{125} I]GLP-1-(7-36)NH₂, peak 2 corresponds to [^{125} I]GLP-1-(9-36)NH₂, and other peaks consist of unidentified [^{125} I]GLP-1 fragments.



reduced insulin response to the secretion of these hormones. This might then result in insufficient insulin secretion in response to a glucose load, yielding diabetic-type symptoms. Interestingly, elevated urinary concentrations of DPP IV have been shown in noninsulin-dependent diabetic patients (48–50). As this elevation preceded the onset of microalbuminuria that is associated with nephropathy in diabetics, it was suggested that the alteration of proximal tubules occurs before pathological changes in glomeruli and brings about enzyme leakage from the brush border. If this enzyme dissociation is a response to the elevated glucose levels, then it is possible that the maintenance of high plasma glucose concentrations could also result in DPP IV dissociation from other tissues, such as endothelium, and lead to elevated levels in the plasma. This hypothesis is currently being investigated.

In conclusion, a sensitive method has been developed to monitor the degradation of [^{125} I]GIP and [^{125} I]GLP-1-(7-36)NH₂. Separation of the cleavage products of [^{125} I]GIP-(1-42) and [^{125} I]GLP-1-(7-36)NH₂ *in vitro* and *in vivo* combined with the action of purified DPP IV, enzyme inhibitors, and serum from DPP IV-deficient rats indicate that DPP IV is a principal enzyme resulting in the degradation and biological inactivation of GIP and tGLP-1. The results suggest that degradation of GIP and tGLP-1 can be reduced by including diprotin A, aprotinin, and bacitracin, in addition to maintaining samples at 4 C. It also seems probable that the biological activity of exogenous GIP and tGLP-1 could be extended by using DPP IV-resistant analogs. The ubiquitous nature of this enzyme, including epithelial cells of the intestine, might explain the isolation of considerable quantities of GIP-(3-42) from natural GIP preparations. This and smaller enzyme fragments of GIP along with biologically inactive tGLP-1 fragments undoubtedly contribute to the overall immunoreactivity measured by GIP or tGLP-1 RIAs currently

in use. New assays, therefore, should be developed to re-evaluate the secretory patterns of biologically active GIP and tGLP-1 under normal and pathophysiological conditions.

Acknowledgments

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